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INVESTIGATING ADIPOSE TISSUE TURNOVER IN HUMANS USING RADIOCARBON DATING

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Cover illustration: Illustration of a mysterious universe of adipocytes. We should never stop exploring.

Investigating Adipose Tissue Turnover in Humans Using Radiocarbon Dating
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my family, to my dearest friends, to my wife, to my son. To those who shaped me into who I am. To everyone I love.

Time is never time at all if you can ever leave without leaving a piece of youth.

And our lives are forever changed, we will never be the same. The more you change the less you feel.

- Billy Corgan

ABSTRACT

Obesity, defined as an excessive accumulation of body fat, is considered one of the major health challenges facing the world today. Adipocyte and lipid turnover determine the number and the size of fat cells, respectively. Therefore, understanding the dynamics of adipocyte and lipid turnover will provide important insights into the factors regulating the size of the fat mass. Adipose tissue is not uniformly distributed throughout the body, and the regional differences in turnover may contribute to the link between body fat distribution and increased risk of metabolic complications. Several factors such as sex and age are also known to impact fat accumulation, however, their influence on adipose tissue turnover dynamics are unclear. In this thesis, we provide a comprehensive analysis of age and turnover dynamics of both adipocytes and lipids in human subcutaneous (scWAT) and visceral (vWAT) adipose tissue, by measuring the integration of ^{14}C derived from nuclear bomb tests into genomic DNA and lipids, across the lifespan. The effects of fat mass distribution, sex, age and BMI were investigated for their influence on adipose tissue turnover, as well as interventions such as weight loss over time.

In **Paper I**, we examined the differences in lipid turnover in scWAT and vWAT in individuals with a range of BMIs. While scWAT lipid age and storage capacity were increased in overweight and obese individuals, lipid age in vWAT was increased only in the excessively obese individuals and was associated with a reduced lipid removal rate. Moreover, in central or visceral obese individuals, lipid turnover was selectively increased in vWAT. Metabolically unhealthy obese individuals with small scWAT adipocytes exhibited reduced lipid turnover. In conclusion, excess body fat mass results in a depot-specific reduction in lipid turnover. Increased lipid turnover in vWAT and/or decreased lipid turnover in scWAT may contribute to metabolic complications of overweight or obesity.

In **Paper II**, we explored lipid turnover dynamics in adults who were followed for up to 16 years (mean follow-up time 13 years) or 5 years following significant weight loss. Lipid removal rate decreased with aging, and a failure to reciprocally adjust for lipid uptake rate resulted in weight gain. Substantial weight loss was primarily driven by a reduced rate of lipid uptake. Surprisingly, individuals with a low baseline lipid removal rate were more likely to maintain the weight loss 5 years following weight-loss surgery. Taken together, these findings identify lipid turnover as an important regulator of long-term obesity development and weight loss maintenance.

In **Paper III**, we compared the age of adipocytes and lipid in human scWAT and vWAT. vWAT adipocytes were found to be older than scWAT adipocytes, suggesting regional differences in the adipocyte death rate. Subject sex, age and body fat mass also had a significant impact on adipocyte age. Females had a slower death rate of adipocytes than males, and age and BMI inversely correlated with the adipocyte death rate. Lipid removal rate was also found to be lower in females than males, and decreased with aging in both depots. In

summary, these results identify adipocyte and lipid removal rates as important factors contributing to regional differences in fat distribution, and that sex, age and BMI-dependent differences in turnover dynamics influence one's risk developing obesity-associated metabolic complications.

Taken together, this thesis provides a deeper characterization of adipocyte and lipid age and turnover dynamics in humans. Furthermore, it highlights regional differences in adipocyte and lipid turnover and the important link to total fat mass accumulation and distribution, in both lean and obese individuals, females and males, and the young and the elderly.

LIST OF SCIENTIFIC PAPERS

I. Spalding K. L., Bernard S., Näslund E., Salehpour M., Possnert G., Appelsved L., **Fu K.-Y.**, Alkass K., Druid H., Thorell A., Rydén M., Arner P. (2017). Impact of fat mass and distribution on lipid turnover in human adipose tissue. Nat Commun **8**: 15253.

II. Arner P., Bernard S., Appelsved L., **Fu K.-Y.**, Andersson D. P., Salehpour M., Thorell A., Rydén M., Spalding K. L. (2019). Adipose lipid turnover and long-term changes in body weight. Nat Med **25**(9): 1385-1389.

III. **Fu K.-Y.**, Jones C. V., Krämer N., Salehpour M., Possnert G., Sirimanna P., Taylor C., LePage P., Suen M., Martin D., Bernard S., Spalding K. L. The Dynamics of Fat Cell and Lipid Turnover in Omental and Subcutaneous Adipose Tissue in Adult Humans. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

BMI	Body fat mass
WAT	White adipose tissue
UCP-1	Uncoupling protein-1
SVF	Stromal vascular fraction
AP	Adipocyte progenitor
scWAT	Subcutaneous white adipose tissue
vWAT	Visceral white adipose tissue
TG	Triglyceride
FFA	Free fatty acid
TNF-α	Tumor necrosis factor alpha
NEFA	Non-esterified fatty acids
LPL	Lipoprotein lipase
DNL	De novo lipogenesis
ACC	Acetyl-CoA carboxylase
ATGL	Adipose triglyceride lipase
DAG	Diacylglycerol
HSL	Hormone-sensitive lipase
MAG	Monoacylglycerol
VLDL	Very low-density lipoprotein
PKA	Protein kinase A
OM	Omental
scABD	Subcutaneous abdominal
PHH3	Phosphor-histone H3
PCNA	Proliferating cell nuclear antigen
AMS	Accelerator mass spectrometry
WHR	Waist-to-hip ratio
MIMS	Multi-isotope image mass spectrometry

1. INTRODUCTION

1.1 Obesity and adipose tissue

1.1.1 Obesity: a global health issue

Obesity is a noncommunicable and preventable disorder defined by excessive body fat accumulation and impaired health. Rates of obesity are increasing to epidemic proportions in most countries and obesity now represents a major worldwide health challenge[1]. Body mass index (BMI) is a simple and commonly used index of weight-for-height to classify normal weight, overweight, and obesity in adults. According to the World Health Organization (WHO), normal weight is defined as a BMI of less than 25 kg/m^2, overweight as 25 to 29.9 kg/m^2, and obesity as equal or higher than 30 kg/m^2. In 2016, an estimated 1.9 billion adults worldwide were classified as overweight and, of these, over 650 million were obese. These numbers have nearly tripled since 1975. If the current trend continues, an additional 20% of people will be obese by 2030[2, 3]. Obesity is linked to an increased risk of disease, such as diabetes, fatty liver disease, and cardiovascular disease, which together are referred to as the metabolic syndrome[4-7]. Due to the vast number of obesity-associated pathologies, the need to advance our understanding of body fat mass regulation is growing.

1.1.2 Adipose tissue architecture

Adipose tissue is composed of several different cell types (**Figure 1.1**). Mature adipocytes, the lipid-storing cells, are the predominant cell population and are distinct both morphologically and functionally, from the other cell types in adipose tissue. Mature adipocytes are further divided into three types by based on their distinct functional and structural properties: white, brown, and beige. White adipocytes which make up the majority of the volume and weight of white adipose tissue (WAT), act as the main energy storage site and endocrine centre. White adipocytes have a large unilocular lipid droplet which occupies 95% of the cell volume and thereby determines the cell size, ranging from 20 to 200μm in human. Brown adipocytes are smaller, mitochondria-rich, and contain multilocular lipid droplets. They are specialized to dissipate energy by heat generation through the action of the brown adipose tissue-specific protein, uncoupling protein-1 (UCP-1), in the mitochondria. Beige adipocytes have gained recognition more recently and remain controversial. While some suggest they arise from a unique progenitor population[8], other evidence indicates that they may be trans-differentiated from white adipocytes[9]. Beige adipocytes share some similar characteristics of brown adipocytes, such as the ability to dissipate heat, and thus are also of interest as an anti-obesity therapeutic target to burn rather than store energy[9]. While brown and beige fat remain therapeutically interesting, by far the majority of fat in the human body is white fat, which forms the focus of this thesis.

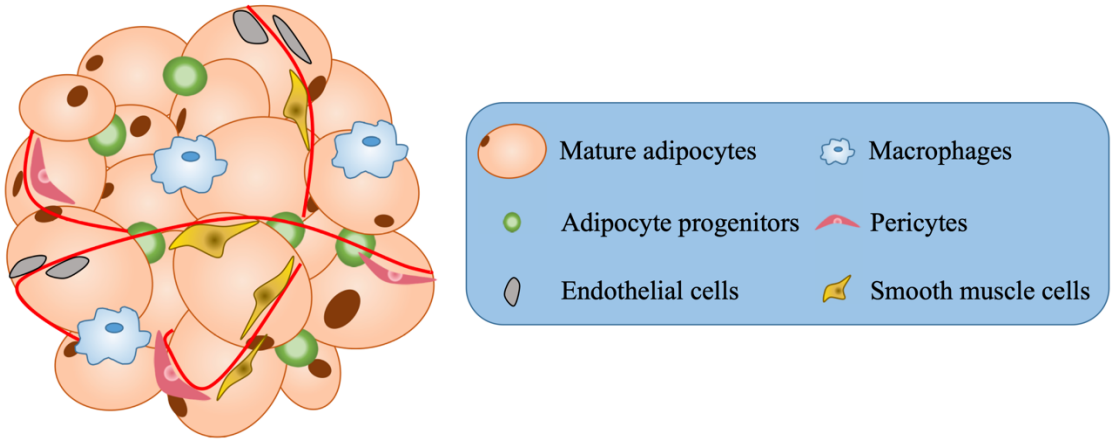

Figure 1.1. Major cell types in white adipose tissue.

In addition to mature adipocytes, WAT is composed of multiple cell populations including endothelial cells, smooth muscle cells, macrophages, pericytes, and adipocyte progenitors (APs) – collectively called the stromal vascular fraction (SVF). The SVF was isolated for the first time by Rodbell in 1964 using collagenase digestion and centrifugation, thereby advancing the knowledge of adipose tissue composition and physiology[10]. Building on this method for separating adipocytes from the SVF, Hollenberg et al. used the incorporation of tritiated thymidine into the genomic DNA of the SVF to identify the source of newly generated mature adipocytes[11]. The finding that an adipogenic progenitor population resided within the SVF laid the path for the extensive research into the regulation of WAT expansion. Because mature adipocytes are considered to be post-mitotic and no longer able to proliferate *in vivo*, the recruitment of newly formed adipocytes is believed to originate solely from the proliferation and differentiation of these APs[12]. Studies have explored the molecular signature of white APs in mice and the niche in which they reside[13, 14]. A subset of SVF cells intimately associated with the adipose vasculature were defined as the "adipose stem cells"[12]. Similarly, studies by Zimmerlin et al. suggested that pericytes resident in adult human WAT constitute the AP population[15]. This supports the concept that mature adipocytes arise from progenitors residing in the adipose tissue SVF.

Adipose tissue is distributed throughout the body in regions described as subcutaneous (scWAT) or visceral (vWAT). The major depots of scWAT include abdominal, truncal, gluteo-femoral and mammary subregions. vWAT includes omental, mesenteric and retroperitoneal depots.

1.1.3 Adipose tissue function

Adipose tissue is a complex and multicellular organ that influences the function of many organs throughout the body via the release of metabolites and adipokines. Historically, the essential function of WAT was considered thermal insulation, mechanical protection and energy storage / release. Based on hormonal and energetic cues, the adipose tissue acts as a site for triglycerides

(TG) storage by lipid uptake and esterification, or a source of free fatty acid (FFA) released by lipolysis. Since the beginning of the 1990s, there has been a profound shift in the way we view adipose tissue due to the discovery of a number of potent adipokines such as leptin, tumor necrosis factor alpha (TNF-α) and adiponectin[9, 16-18]. Over 600 adipokines have been described in different proteomic reports and adipose tissue is now considered to be an important endocrine organ[16-18]. While most adipokines exert their effects locally on the adipose tissue itself via paracrine signalling, others have endocrine function and are released into the circulation and act on distant tissues[19-22]. The release of leptin, which suppresses appetite via the hypothalamus and increases thermogenesis via sympathetic nerve activity, exemplifies how adipokines impact energy balance and body weight[23].

1.1.3.1 Lipogenesis and lipolysis

The primary function of white adipocytes is to act as lipid-storing cells. Lipogenesis is the process of energy storage in the form of TG, by utilizing fatty acids mainly derived from meals. When energy is in excess, TGs are synthesized by the intestine and liver and packed as lipoproteins and transported to adipose tissues. TGs are hydrolysed into non-esterified fatty acids (NEFA) via the action of lipoprotein lipase (LPL) in adipose endothelial cells. NEFA then enters adipocytes where they are subsequently esterified and stored[24, 25]. Whilst most stored lipids come from exogenous fatty acids, fat cells are capable of synthesising lipids from carbohydrates via de novo lipogenesis (DNL). Generally, DNL includes i) fatty acid synthesis from acetyl-CoA through the action of acetyl-CoA carboxylase (ACC), and ii) TG synthesis, where fatty acids and glycerol 3-phosphate form TG[26-28].

The removal of fatty acids from adipocytes is a stepwise catabolic reaction termed lipolysis. The lipolytic machinery consists of three major enzymes. Firstly, adipose triglyceride lipase (ATGL) hydrolyses TG into diacylglycerol (DAG) and one NEFA. In the second step, hormone-sensitive lipase (HSL), which displays hydrolase ability towards both TG and DAG but is preferential to the latter, converts DAG into monoacylglycerol (MAG) and one NEFA. Monoacylglycerol lipase completes the process by hydrolysing MAG to generate glycerol and the third NEFA[29, 30]. Overall, the stored TGs are hydrolysed into NEFAs and glycerol and released into the circulation. Circulating fatty acids are taken up by other tissues under energy demand and utilized for ATP generation through β-oxidation. The excess FFAs travel back to the liver to be used as a source of very low-density lipoprotein (VLDL) production[31]. However, some FFAs do not leave the adipocytes, or are directly taken up by the adjacent adipocytes, termed direct FA uptake, to reform TGs[32, 33]. The steps of lipogenesis and lipolysis are summarised in **Figure 1.2**.

Both lipogenesis and lipolysis occur in response to various hormones and energy balance cues. Insulin upregulates lipogenesis by facilitating glucose uptake as the source of DNL as well as enhancing the expression and activity of LPL in adipose tissue[34, 35]. Although adipocytes

perform a basal level of lipolysis, this process is largely driven by β-adrenergic signalling. Catecholamines, such as epinephrine and norepinephrine, bind to β-adrenergic receptors (ADRB1 and ADRB2) on the adipocyte cell membrane to promote cAMP production and protein kinase A (PKA) activation. PKA phosphorylates rate-limiting HSL which in turn initiates the lipolytic process[29, 36]. Whilst facilitating lipogenesis, insulin also acts as an antilipolytic hormone, inhibiting ATGL and HSL activities[37-39]. Other mediators, such as TNF-α, glucocorticoids and natriuretic peptides, also have physiological influences on lipid mobilisation[29, 30, 40].

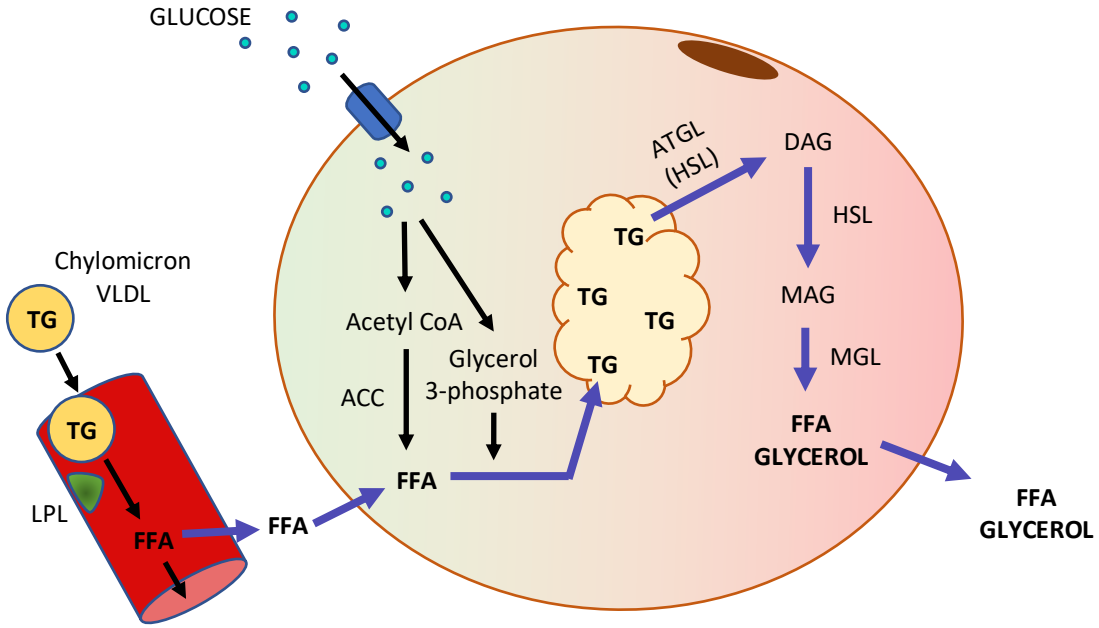

Figure 1.2. A brief schematic representation of lipogenesis and lipolysis in adipocytes.

1.1.4 Depot-specific differences

WAT develops in different body locations and exhibits remarkable heterogeneity in cellular composition, tissue function and response to hormones. These differences may be due to the intrinsic characteristics of WAT depots (e.g. differences in AP gene signature and differentiation capacity) but also the unique microenvironment of each depot, such as its innervation and circulation[41, 42]. Although vWAT only accounts for about 20% of total body fat, its venous drainage flows into the portal circulation and immerses the liver with its metabolites and adipokines, making vWAT strongly linked to an adverse metabolic profile. On the other hand, because scWAT can expand outward with minimal anatomic constraint, it serves as a sink to store potential cytotoxic fatty acids and has less, or even an inverse, correlation with metabolic disease[43-45]. As such adipose tissue in these regions is now recognized as physiologically distinct. Obese individuals who preferentially store excess lipids in vWAT have a higher risk for metabolic diseases than those with a similar BMI but who accumulate lipid in their scWAT. This suggests that the susceptibility to obesity-associated metabolic

complications is not solely dependent on the amount of total body fat, but also regional body fat distribution and the ability of scWAT to expand[41, 45].

1.1.4.1 Cellularity and morphology

Despite notable inter-individual variation, adipocyte size is generally smaller in vWAT than scABD, although the difference becomes less obvious in obese individuals[42, 46]. The SVF accounts for approximately 50% of total cell number in WAT[47]. SVF density is higher in omental (OM) fat, one of the major visceral fat depots, compared to the subcutaneous abdominal (scABD) fat depot[48, 49]. However, there are more SVF cells in scABD as it is larger in tissue size than the OM fat depot in normal-weight individuals under physiological condition. Moreover, not only is AP cell number higher, but proliferation and differentiation abilities are also higher in scABD than OM depot[48-51].

1.1.4.2 Lipid metabolism

WAT stores more than 95% of total lipids, and the uptake and release of FAs are highly regulated in a depot-specific fashion. FA uptake from meals, as well as direct FA uptake, were shown to be higher in the OM fat depot compared to the scABD fat depot per unit fat mass[52-54]. LPL activity, the key element for lipid uptake and TG synthesis, was shown to be higher in scWAT than OM fat with an exception in obese men[55, 56]. With higher lipolytic β-adrenergic receptor expression and lower insulin-induced antilipolytic sensitivity[56-59], OM adipocytes exhibit more of a lipolytic-prone status compared to scABD. Taken together, these differences in lipid metabolism contribute to the variation in preferential lipid storage sites[60, 61].

1.1.4.3 Adipokine secretion

WAT secretes different hormones and bioactive molecules that exert their effects in an auto-, para-, and endocrine manners to regulate systematic metabolism. Leptin and adiponectin are the most studied adipokines, which regulate appetite, lipid metabolism, immune function and reproduction[42, 62]. There are depot-specific differences in adipocyte protein production also. For instance, the expression of proinflammatory adipokines, including IL-6, IL-8 and MCP-1, are higher in vWAT[60], contributing to the link between visceral adiposity and metabolic health. Omentin, which acts as an insulin action modulator, is a visceral specific adipokine which is only synthesized and released by the OM depot[63].

1.2 Fat mass regulation

1.2.1 Adipose tissue expansion

One of the features that makes WAT a unique organ is its remarkable plasticity. WAT changes its mass by regulating the size and/or the number of adipocytes. During a state of overnutrition, two mechanisms are involved in WAT expansion: the enlargement of fully differentiated adipocytes, termed hypertrophy; and the proliferation and differentiation of APs, termed

hyperplasia. In principle, WAT first expands by increasing adipocyte size until they reach a critical threshold of lipid-filling. The "full" adipocytes release signals that induce AP proliferation/differentiation to accommodate the excess lipid influx[64]. Previous studies have reported a linear-curve relationship between adipocyte volume and body fat mass, demonstrating that increases in fat mass cannot be explained by an increase in adipocyte volume alone. The nature of the curve suggests the increase in adipocyte number occurs in the later stage of obesity development[65-67] (**Figure 1.3**). The precise timeline of the fat mass expansion mechanisms during overfeeding, however, remains unclear. As an individual continues to gain weight, scWAT, the main fat storage site, can no longer store more lipid through increasing adipocyte size or number, and ectopic lipid deposition occurs. Ectopic lipid deposition in non-scWAT tissues such as vWAT, skeletal muscle, liver and pancreatic beta cells has lipotoxic effects, including insulin resistance, inflammation and apoptosis[42, 68, 69]. Similarly, lipodystrophy, defined as the inability to store lipids in scWAT, can also lead to ectopic deposition and a similar metabolic outcome as in obesity[42, 70].

The mechanism of fat mass expansion has an impact on metabolic profile. Increased adipocyte size is associated with other risk factors for type 2 diabetes, such as insulin resistance and glucose intolerance, independent of fat mass or distribution[67, 71-73]. Using the linear-curve relationship between adipocyte volume and body fat mass, Arner et al. defined a "relative morphology value" as the difference between the measured mean adipocyte volume and the expected adipocyte volume for any given body fat mass[74]. Individuals with positive morphology values are categorized as undergoing hypertrophic expansion, whereas individuals with a negative value are considered hyperplastic (**Figure 1.3**). Furthermore, the authors showed that women with hypertrophic expansion had a more adverse metabolic profile than women with the same body fat mass level but who exhibited hyperplastic expansion. In line with this study, others have reported that WAT from individuals with metabolic syndrome is characterized by hypertrophic adipocytes, hypoxia, apoptosis and inflammation, and associated with abdominal obesity, insulin resistance, and ectopic lipid deposition[75-78]. On the other hand, WAT from metabolically healthy individuals had a higher vessel density with relatively small adipocytes[75-79] (**Figure 1.3**), indicating hyperplastic expansion may be a relatively protective expansion mechanism. Interestingly, one study reported that the loss of both upper and lower body scWAT mas was only attributed to a reduction in adipocyte size, not number[80]. The precise timeline of fat mass change mechanisms during weight gain and loss warrants further long-term longitudinal studies.

1.2.2 Depot-specific fat mass expansion and determinants of fat mass and fat distribution

Total fat mass and BMI positively correlate with cardiometabolic diseases, however, body fat distribution better predicts metabolic complications and is of clinical relevance[41, 45]. Different adipose tissue depots are distinct with respect to their cellular composition and function and

therefore play unique roles in metabolic control. Overnutrition leads to adipose tissue enlargement in both scWAT and vWAT. However, vWAT depots reach their expansion limitation early in the development of obesity, while scWAT depots grow steadily as obesity

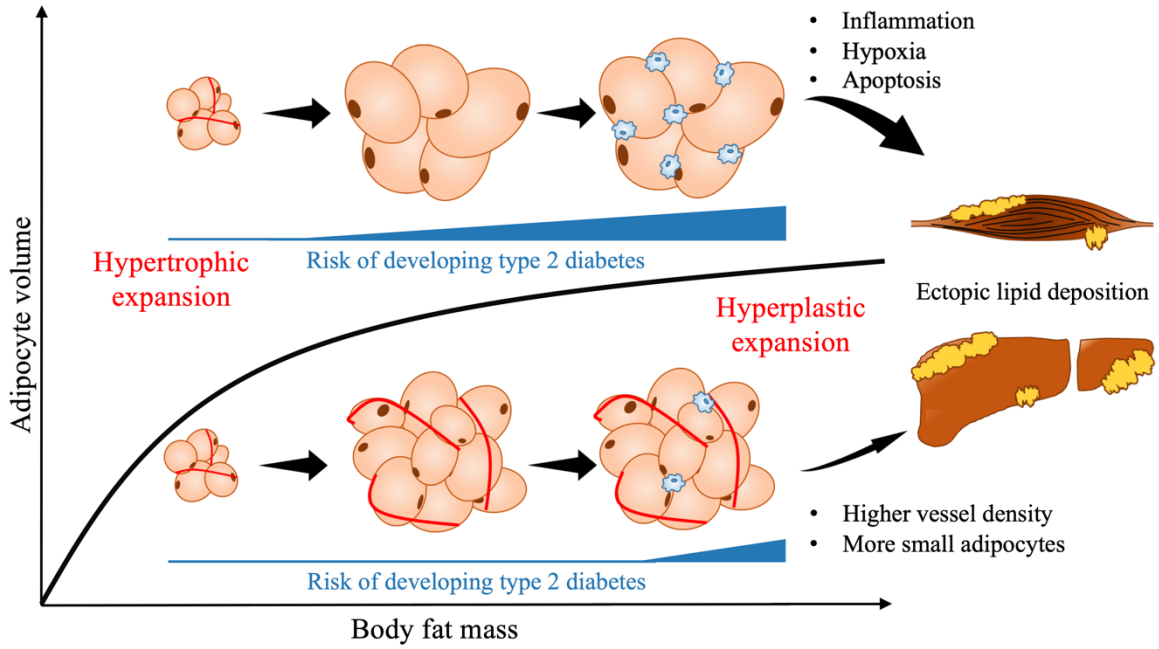

Figure 1.3. Hypertrophic versus hyperplastic fat mass expansion (Figure adapted from Hepler et al., 2016)[81].

develops[82]. Individuals with central obesity, or apple-shaped obesity, accumulate excess fat in the abdominal region, especially vWAT, and have a higher risk for cardiometabolic disease, such as atherosclerosis and diabetes[41, 83]. On the contrary, lower body or pear-shaped obesity, where individuals preferentially store fat in the gluteofemoral areas, is associated with lower risk, or even protection, from metabolic disease[84, 85]. The different outcomes of regional fat accumulation may be attributed to differences in depot-specific fat mass expansion mechanisms. vWAT generally expands through an increase in adipocyte size, whereas scWAT enlarges by both an increase in fat cell size and number[41, 86]. This greater expandability, which may result from the greater proliferation and differentiation capacities of APs in scWAT depots, may explain the protective role of scWAT[41, 50, 86]. However, scWAT depots are not all uniform. Gluteofemoral fat tends to expand in a relatively hyperplastic manner during short-term overfeeding, whilst upper body scWAT expands via hypertrophy[86], thereby explaining the better metabolic outcomes of a pear body shape. Taken together, the adipogenic capacity of APs in scWAT leads to greater expandability potential, enabling scWAT to act as a protective lipid storage sink to minimize ectopic lipid deposition in vWAT and other ectopic sites.

Sex differences in fat distribution, adiposity, and lipid metabolism add another layer to the complexity of fat mass regulation. Females generally have higher levels of adiposity and primarily store lipid in scWAT depots (especially in the lower body) whilst males have more

lean mass but preferentially accumulate fat in visceral depots. This makes sex itself a strong predictor for metabolic disease risk[87-90]. The factors regulating sexual dimorphism have not been fully elucidated. Studies have shown that sex-dependent total fat mass and fat mass distribution differences diminish at later stages in life, as vWAT mass increases in postmenopausal women[91]. Moreover, women with polycystic ovary syndrome, characterized by a hyperandrogenic state, frequently exhibit abdominal obesity[92]. These results have brought much attention to the important role that sex hormones play in fat mass regulation, however, the mechanisms involved remain unclear. Additional factors are also likely involved given that sex-dependent differences are already present before puberty[93]. By using the "four core genotypes" mouse model, Chen et al. showed that the number of X chromosomes also contributes to the sexual dimorphism. In this model, mice were gonadectomized to remove the effects of gonadal hormones while maintaining an XX or XY sex chromosomal identity. The Y chromosome, more specifically, the *Sry* gene located on the Y chromosome, initiates differentiation of the testes. Mice that have a *Sry*-deleted Y chromosome (XY^-) develop ovaries, rather than testes. Conversely, an *Sry* transgene inserted onto an X chromosome is sufficient to convert XX female mice to gonadal males. By these four genotypes, the authors reported that mice with two X chromosomes had up to two-fold higher adiposity, regardless of the gonadal sex, compared to mice with XY chromosomes. They further produced mice carrying XO and XXY chromosome complements and revealed that the adiposity difference between XX and XY is attributed to the number of X chromosomes rather than the effect of the Y chromosome[94]. This result suggests that a subset of protein-coding genes, which are located in the X chromosome, may contribute to sex differences in fat mass regulation and represent candidates for future studies into factors impacting adiposity.

The amount and distribution of adipose tissue also changes throughout life. In general, fat mass increases with advancing age due to a chronic positive energy balance, reduced physical activity and lower metabolic rate. The increase in fat mass is more pronounced in the abdominal region, where males persistently accumulate fat, while females shift their preferential lipid storage site from lower body fat depots to abdominal depots[95, 96]. These age-related alternations in body fat mass and distribution lead to a higher risk of metabolic complications in the elderly, including ectopic lipid deposition, insulin resistance and chronic systematic inflammation[97, 98]. Understanding the determinants of adipose tissue expansion and distribution, such as sex and age, and the underlying mechanisms will provide targets for developing novel therapeutic approaches which better address obesity-associated metabolic complications.

1.2.3 Adipose tissue turnover in the regulation of fat mass and fat distribution

Body fat mass is determined by the number and size of adipocytes. The balance between the proliferation and differentiation of APs into mature adipocytes and the death rate of existing adipocytes determines adipocyte number. If the generation rate is higher than the death rate of adipocytes, fat cell number grows. Similarly, the storage and removal rates of lipid within

adipocytes control adipocyte size. An increase in adipocyte size occurs when the lipid storage rate is higher than the removal rate. Therefore, the balance between synthesis and breakdown (i.e. turnover) of adipocytes and lipids is crucial for fat mass regulation (**Figure 1.4**).

Cross sectional studies in humans have shown that both adipocyte size and number increase during childhood and adolescence but that the number reaches a plateau and remains constant afterwards[65, 66]. Individuals obese since childhood, however, may have twice the number of adipocytes prior to adulthood than lean individuals[65]. Longitudinal studies, where individuals with or without weight change are followed for many years, will provide important insight into how adipocyte number changes throughout life. Short-term weight change studies, however, also provide insights into how adipocyte number is regulated. Salans et al. reported that only fat cell size, not number, changed in scWAT depots in healthy non-obese men during 3 - 4 months of weight gain and subsequent weight loss periods, supporting the conclusion of a tight regulation of adipocyte number in adulthood[99]. Total adipocyte number in this study was, however, calculated as total body fat mass divided by average adipocyte size across three scWAT depots. By pooling adipose depots, subtle yet significant differences in fat cell number as a function of weight gain and loss, may have been lost[46].

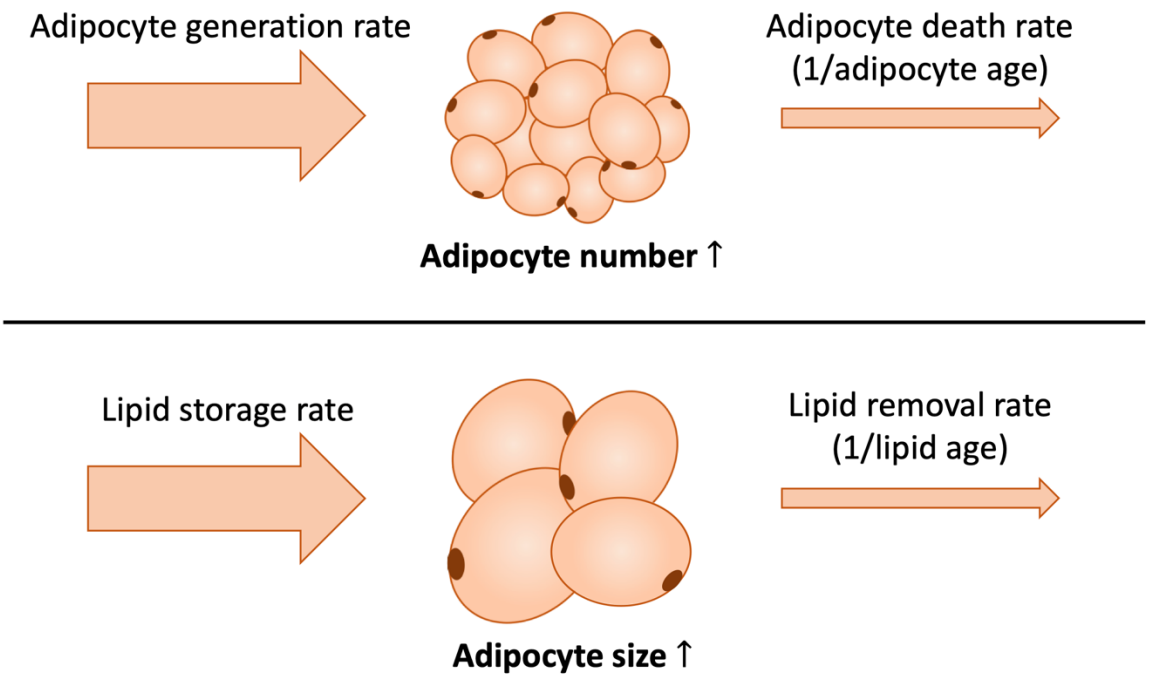

Figure 1.4. Adipocyte and lipid turnover regulate adipocyte number and size.

In a more recent study, the regional differences in fat mass expansion in women following an eight-week period of weight gain were revealed. Here the authors demonstrated that an increase in adipocyte number only occurred in lower body fat, but not in the scABD depot[86]. In a similar experimental setting, Singh et al. reported that adipocyte number remained constant after a

period of weight loss[80]. These results concur with other studies that show once a higher adipocyte number is established, weight loss does not result in a loss of gained fat cells[65], leading people to propose a "thrifty genotype" theory. The "thrifty genotype" theory suggests that humans have gone through a positive evolutionary selection for genetic mechanisms that favor energy storage to survive ancient times of famine. The combination of the modern sedentary lifestyle, plus long-term overnutrition, now contribute to the obesity epidemic we face today[100, 101].

Regional differences in adipose tissue lipid kinetics may contribute to the depot-specific differences in adipocyte size. As mentioned above, mean adipocyte size in the scABD depot is larger than the OM depot in general. In support of this, studies have shown that OM adipocytes not only exhibit greater responsiveness to lipolytic adrenergic agonists in both sexes due to higher ADRB1 and ADRB2 expression[56-58], they are also less sensitive to the antilipolytic effect of insulin[59]. Taken together, these characteristics push OM adipocytes into a lipolytic state and increase FA efflux via the portal vein to the liver, contributing to the adverse metabolic effect of the OM depot[56-58]. Other factors, such as sex, are also involved in lipid metabolism and adipocyte size regulation through complex interactions[102]. For instance, men have larger adipocytes, especially in vWAT depots, than women but this size difference diminishes in obese individuals[58, 103] (reviewed in Ref. 100).

1.3 Current knowledge of human adipose tissue turnover dynamics

1.3.1 Methods to study cell and lipid turnover

Due to ethical and technical difficulties in studying cell turnover in humans, most of our understanding of cell turnover is translated from animal studies. This is often from rodents, which may not be an ideal model for humans who can live for up to a century and potentially have a greater need for cell replacement throughout the life span. Traditional methods often involve the detection of cell proliferation markers such as Ki-67, phospho-histone H3 (PHH3) and proliferating cell nuclear antigen (PCNA)[104-106]. Although these methods provide good estimates of the number of cells in cell cycle at a given point in time, they fail to give information concerning the fate of the labeled cells and offspring. Whether the progeny will mature and integrate into tissues, or when the labeled cells were born cannot be concluded. Another strategy to estimate cell generation is via the administration of labeled nucleotide analogues, such as ^{3}H-thymidine, 5-bromo-2'-deoxyuridine (BrdU) and 5-ethynyl-2'-deoxyuridine (EdU), which are incorporated into newly synthesized DNA during S-phase[107, 108]. These prospective labeling methods have been useful for revealing cell generation in experimental animal models. It is, however, difficult to detect rare events (i.e. cells with slow turnover) and their use in humans is limited due to potential cytotoxicity of labeled nucleotides for long-term chasing. Meanwhile, most of the lipid metabolism studies that have provided important insights into lipid biology have been performed using adipose tissue biopsies in *in*

vitro environments. The interpretation of these studies is hampered by the absence of the WAT's natural milieu, for instance, the local adipocyte production of the antilipolytic compound, adenosine[109]. Hence, *in vivo* approaches are necessary to accurately investigate adipocyte and lipid turnover mechanisms.

Ravussin and colleagues assess both adipocyte and lipid turnover kinetics *in vivo* by measuring the short-term (8 weeks) incorporation of the stable isotope deuterium from heavy water (^2H_2O) into adipocyte DNA and lipid[110-112]. While these studies provide powerful insights into understanding adipose tissue turnover during different interventions, such as dietary or pharmacological treatments, over short time periods, they do not give information over the lifetime of an individuals. Retrospective ^{14}C dating, a method developed recently by our group, provides information regarding long-term cell turnover dynamics by analyzing the integration of ^{14}C derived from nuclear bomb tests into genomic DNA and lipids[65, 113, 114] (for background and a detailed description on radiocarbon dating, see **Methods 3.1**). This method allows for the retrospective analysis of adipocyte/lipid turnover in humans across the lifespan of an individual.

1.3.2 Current studies addressing *in vivo* measurement of human adipose tissue

Studies have shown that early phases of overfeeding or weight loss in adults predominantly modifies the size of adipocytes and not the number[80, 99]. For decades, such observations supported the notion that adipocytes are terminally differentiated, non-turning over cells, which respond to nutritional changes only by regulating lipid storage and removal. Whether mature adipocytes were generated from progenitor cells in adulthood was unknown. Work in the Arner and Spalding laboratories, however, showed that while adipocyte number in adults may remain constant, there is a continuous turnover of mature adipocytes where the adipocyte generation rate matches the death rate[65]. The estimates of adipocyte turnover in humans vary greatly with two different study methods. White et al. assessed adipocyte turnover kinetics in scWAT depots via an eight-week incorporation of deuterium (heavy water). They report that approximately 8% of adipocytes were newly generated during a 8-week labeling period[111]. Moreover, they demonstrated depot-dependent differences in adipocyte generation rate, with higher adipocyte formation in the femoral fat depot compared to scABD depot. Notably, the adipocyte death rate, an important component of adipocyte turnover, was not measured in this study. However, based on the assumption that a person remained weight stable during the experimental period, the adipocyte birth rate was likely balanced by the death rate to indicate the adipocyte turnover rate. Compared to the rapid adipocyte turnover estimated by the heavy water method, we previously reported that approximately 10% of adipocytes in scABD are replaced every year[65]. The carbon dating method measures the age of cells present in the samples and, by extension, their death rates. The heavy water method, on the other hand, measures newly synthesized DNA with deuterium and thus the adipocyte birth rate. Including newborn adipocytes (i.e. differentiated post-mitotic AP), which have a higher turnover rate than mature adipocytes, into measured adipocytes may result in an overestimation of adipocyte birth rates (see Supplementary

Information 3 in **Reference 64**). Possibly, the 7- to 12-fold difference in the adipocyte turnover rates estimated by two methods may represent the excess ratio of APs to mature adipocyte generated, indicating that most proliferating APs do not permanently integrate into mature adipocyte population. One other potential explanation for these differences is the purification of adipocytes isolated from enzymatically digested adipose tissue. Contamination by the highly proliferative SVF cells may result in an over-estimation of the adipocyte turnover rate. However, we have reported adipocyte purification efficacy by our adipocyte isolation protocol is higher than 98%[65]. In support of the role of adipocyte purity being an important factor in fat cell turnover estimates, a recent study by Guillermier et al. adopted the heavy water labelling procedure and used multi-isotope image mass spectrometry (MIMS), an image-based method with subcellular resolution, to detect deuterium[115]. The authors reported an approximately 11% yearly adipocyte birth rate. This adipogenesis measurements are similar to the 10% turnover rate estimated by the carbon dating method, and significantly lower than the birth rates reported from the bulk-measurement of heavy water labelled adipocytes. The sensitivity of different stable isotope detections may also contribute to these different turnover estimations.

Arner et al. provide direct evidence as to the important role that adipocyte turnover plays in different adipocyte phenotypes of the scABD depot[74]. Although the percentage of adipocytes replaced each year was similar in hypertrophic and hyperplastic states, the total number of adipocytes generated per year was 70% less in hypertrophic individuals compared to hyperplastic individuals. There was also an inverse correlation between adipocyte size and adipocyte generation rate (i.e. the higher the hypertrophic degree, the lower the adipocyte generation rate). This suggests that a slower generation rate of adipocytes results in existing adipocytes accumulating more lipids, leading to adipocyte hypertrophy and the associated unfavorable metabolic profile. Strawford et al. also applied the heavy water labeling method to measure TG synthesis and turnover. They estimated that the TG half-life was about six months in scWAT depots[116]. By ^{14}C dating method, we further identified that high TG storage, but low removal rates contribute to fat mass accumulation and obesity development in the scABD depot.

In summary, prospective and retrospective methods to investigate adipose tissue turnover in humans have been established. These estimations, however, do not measure exactly the same thing and, as described above, can result in inconsistencies. The advantage of the carbon dating method is its ability to determine cell and lipid turnover over the entire lifespan of an individual, without the need for administrating labelling compounds. The studies mentioned above have assessed only scWAT depots and any information concerning adipose tissue turnover in vWAT is missing[114].

2. RESEARCH AIMS

2.1. General aim

To provide a comprehensive overview of human adipose tissue dynamics by studying adipocyte and lipid turnover, as a product of sex, aging and total fat mass in both subcutaneous and visceral fat depots.

2.2. Specific aims

I. To compare lipid turnover in scABD and OM fat depots in subjects with a range of body fat masses, and to assess how lipid turnover is altered in individuals with central obesity or who are metabolically unhealthy.

II. To investigate how lipid turnover dynamics change over time, following individuals for up to 16 years. The role of lipid turnover in weight loss maintenance, 5 years following bariatric surgery, was also investigated.

III. To define adipocyte and lipid age in subcutaneous and visceral fat, and examine how depot-specific differences in turnover rate associates with body fat mass distribution. Furthermore, we investigated whether factors including sex, age and total fat mass impacted adipocyte and lipid age.

3. Methods

The methods commonly used in the constituent studies of this thesis are described here.

3.1. Retrospective ^{14}C dating

The average age of human adipocytes and lipids was determined by measuring the ^{14}C content of adipocyte genomic DNA (cell age) and triglyceride (lipid age), respectively. ^{14}C is the least abundant naturally occurring carbon isotope (1 part per trillion of total carbon)[113, 117, 118]. Atmospheric levels have remained relatively stable, with respect to all carbon, for the last several thousand years. However, atmospheric detonations of nuclear weapons during the Cold War (1955-1963) notably increased the concentration of ^{14}C / ^{12}C in the atmosphere[117, 118] (**Figure 3.1**). Even though the tests were conducted at specific locations, the increased atmospheric ^{14}C rapidly equalized around the globe. Since the signing of the Partial Test-Ban Treaty in 1963, atmospheric ^{14}C levels have progressively decreased. The decline, however, is not due to radioactive decay, as the half-life of ^{14}C is 5,730 years, but rather is the result of atmospheric ^{14}C diffusing into the oceans and the biotope. Atmospheric ^{14}C reacts with oxygen to form $^{14}CO_2$, which is incorporated into plants by photosynthesis. By eating plants, and animals that live off plants, the ^{14}C concentration in the human body closely parallels that of the atmosphere at any given point in time.

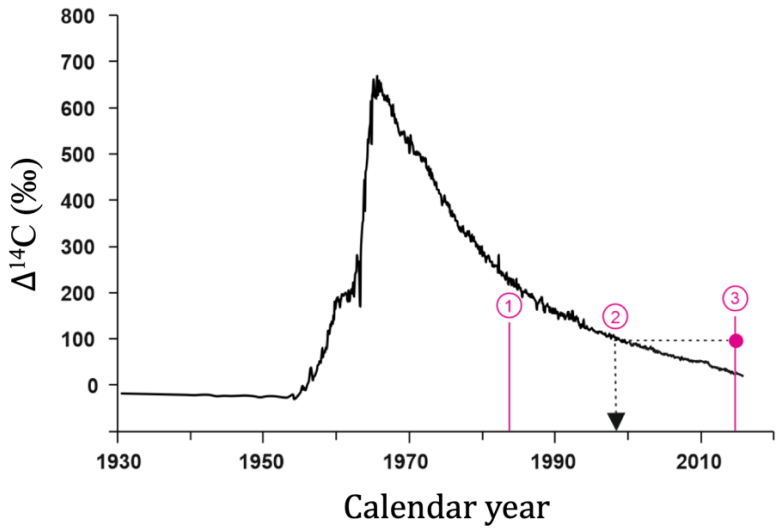

Figure 3.1. Schematic illustration depicting the strategy of ^{14}C birth dating.

By assessing the incorporation of ^{14}C into genomic DNA and lipids, it is possible to determine the age of a cell population and thus the death rate. ^{14}C is integrated into genomic DNA during DNA synthesis. In terminally differentiated cells, that do not undergo further rounds of DNA synthesis, ^{14}C levels in the DNA will remain constant and reflects the date the cell was formed. Thus, the ^{14}C /^{12}C ratio in DNA acts as a date stamp for when an adipocyte was born. Lipid stored in adipocytes, mainly as TG, can also be measured to assess mean lipid age (reflecting

the amount of time lipid is resident in the adipose tissue). Such analysis can provide information as to the removal rate of lipids from the adipose tissue, as well (if total fat mass is known) the amount of lipid stored in the adipose tissue. This allows one to measure the flux of lipids in and out of the adipose tissue and indicates whether alternations in lipid fluxes may contribute to various metabolic diseases.

In Figure 3.1, the black curve shows the atmospheric ^{14}C levels over the last decades. The peak of the curve demonstrates the large addition of ^{14}C during the Cold War. In this example, ① shows a person's date of birth. The pink dot illustrates the ^{14}C data point of a hypothetical sample, where the measured ^{14}C concentration is plotted against the date of collection ③. By comparing the measured ^{14}C concentration with the atmospheric ^{14}C curve (black dashed lines), the average birth year of the hypothetical sample is indicated by ②. The time difference between the date of sample collection and average birth date provides an estimate of mean adipocyte or lipid age. When the measured ^{14}C value ③ approximates the atmospheric levels of the curve, this indicates a contemporary cell or lipid age. Measured values that sit above the curve indicate older cells or lipids.

The detection sensitivity for new cell and lipid generation depends on the steepness of the atmospheric ^{14}C curve between when an individual was born ① and when the new cells or lipids were generated ②. An individual who was born before the dramatic increase in atmospheric ^{14}C levels has the highest sensitivity for detecting cell generation due to the largest difference in ^{14}C levels between the birth date and the cell or lipid generation date. For instance, a measurement in a person who was born 20 years before the increase in ^{14}C will have the highest sensitivity for detecting newly generated cells during early adulthood. In contrast, measurement from a person born after the atmospheric ^{14}C peak will be most sensitive for detecting cells or lipids born more contemporarily. The accuracy of average age estimation, on the other hand, depends on the steepness of the atmospheric ^{14}C curve between cell or lipid generation date ② and the date of collection ③, as well as error margins resulting from measurement error (which depend, for example, on sample handling and sample sizes). The detection limit, when comparing time points on the steepest part of the atmospheric ^{14}C curve with the current sensitivity of accelerator mass spectrometry, is about 1% newborn cells within a population of pre-existing or non-turning over cells. The accuracy, across the atmospheric ^{14}C curve, as evaluated by radiocarbon dating human tooth enamel, is on average +/- 2 years[113].

3.2. Adipose lipid and adipocyte genomic DNA extraction

Adipocytes were isolated from whole adipose tissue samples using a collagenase-based digestion protocol[10] and the purity of the isolation procedure was validated as described previously[65]. Lipid within the adipose tissue is primarily stored in adipocytes, and previous work has shown that the ^{14}C-derived lipid age is comparable when extracted from either whole adipose tissue pieces or isolated adipocytes[114]. Therefore, lipids extracted using an

isopropanol/heptane/H_2SO_4 protocol[119] from either adipose tissue or isolated adipocytes were used. The extracted lipid weight constitutes 70 - 80% of the initial sample weight. Adipocyte DNA was extracted from isolated adipocytes using the phenol/chloroform/isoamyl alcohol extraction protocol described by Liu et al.[120], and modified to maximize the DNA yield while minimizing non-DNA carbon contamination for ^{14}C dating. DNA was measured and purity checked by Nanodrop spectrometer and gel electrophoresis. Potential RNA and protein contamination were also assessed by RNA and protein detecting kits. To minimise the introduction of exogenous carbon whilst maximizing DNA yield and purity, several steps were optimised from the phenol/chloroform/isoamyl alcohol extraction protocol (see Methods in **Paper III**). The extra RNA digestion step and DNA precipitation, rather than the commonly used centrifugation-based collection of DNA pellets, was used in this study to improve DNA purity, while the second precipitation increased DNA yield. All extraction procedures were performed in a carefully cleaned dust-free hood and glassware was prebaked at 450 °C for 4 hours to avoid the introduction of exogenous carbon. The amount of adipose tissue that is required to obtain enough carbon for ^{14}C analysis varies from region to region, depending upon fat cell size. Generally, a minimum of 20 grams of adipose tissue is required for adipocyte ^{14}C analysis and about 0.1 gram of adipose tissue or isolated adipocytes for lipid ^{14}C analysis.

3.3. Accelerator mass spectrometry

To estimate the average age of adipocytes and lipids, the amount of ^{14}C in adipocyte genomic DNA and lipids was measured by highly sensitive accelerator mass spectrometry (AMS). The sensitivity of AMS and the ultra-small sample preparation method required for most biological samples, including adipocyte DNA, have improved considerably during the last few decades, reducing the required biological sample size from about 1 mg of carbon to less than 10 μg[121, 122]. However, single-cell resolution will not be possible given that there is approximately only one ^{14}C atom in the genomic DNA for every 15 cells[113]. ^{14}C levels in cerebellar neuronal DNA was also measured as an internal control for adipocyte measurements. Cerebellar neurons (isolated from human brain samples collected post-mortem) do not turnover across the lifespan[113], and as such, the average age of cerebellar neurons should reflect the date when the person was born. Specific AMS procedures, $\delta^{13}C$ measurements and isotopic fractionation corrections, were performed as detailed in the constituent papers.

3.4. Mathematical modelling

Mathematical modelling was applied to estimate the turnover rates of adipocytes and lipids. In adulthood many tissues replace lost cells with new ones to maintain homeostasis. However, this balance may be different among tissues due to differences in the rate of cell replacement, aging, or under physiological/pathological conditions. For instance, a reduced generation rate of adipocytes may be compensated for by adipocyte hypertrophy during weight gain, or the lipid removal rate may be different between lean and obese individuals. Thus, turnover modelling is aimed at explaining the unique turnover dynamics for a given population of cells, or lipid, and

involves testing a range of scenarios and fitting these scenarios to experimental data obtained from measuring samples that cover the range of the bomb curve. Thus, the aim is to find a turnover rate scenario that best explains the measured ^{14}C levels[123]. The death rate of adipocytes represents the fraction of adipocytes replaced each year and is the inverse of adipocyte age (1 / adipocyte age). As such, a high adipocyte age reflects a low adipocyte death rate. Similarly, the lipid removal rate (K_{out}) indicates the fraction of lipids replaced each year and is the inverse of mean lipid age (K_{out} = 1 / lipid age). If total fat mass is known, the net lipid intake can be determined (K_{in} = kg of fat mass per year. The amount of lipid in kg stored in the adipose tissue each year), reflecting the lipid storage capacity. The modelling methods for adipocytes and lipids are described in detail in the constituent papers.

Atmospheric ^{14}C levels across the two hemispheres correlates, but lags in the Southern Hemisphere (i.e. the ^{14}C levels in the Southern Hemisphere never reach the same height as the Northern Hemisphere at the peak of nuclear testing, but the ^{14}C levels in the Southern Hemisphere are slightly higher than those in the Northern Hemisphere in the last decades). For samples formed in the last 20 year, this slight shift in atmospheric abundance can produce an overestimation of age if samples from the Southern Hemisphere are analysed using the curves calibrated from the Northern Hemisphere measurement. We noticed a 6-month to 1.5-year difference in estimated ages between Northern and Southern Hemispheres. No published data for Southern Hemisphere atmospheric ^{14}C levels after 2006 were available at the time of analysis (August 2020). However, we were kindly provided by Quan Hua (ANSTO, Australia, personal communication) recent Southern Hemisphere data, running up to the end of 2017. Thus, for Northern Hemisphere samples in **Paper I** and **II**, we used the Schauinsland dataset[124]. The updated Southern Hemisphere ^{14}C dataset was used for the analysis in **Paper III**.

4. Results and discussion

4.1. Paper I

Impact of Fat Mass and Distribution on Lipid Turnover in Human Adipose Tissue

4.1.1. Summary of results

Adipocyte size is determined by the balance between lipid storage (K_{in}) and removal (K_{out}), termed lipid turnover. Different lipid turnover rates in scWAT and vWAT may contribute to depot-specific fat cell size differences, potentially contributing to metabolic complications in overweight and obese individuals. A previous study has shown that scABD lipid storage and removal rates are altered in obese individuals compared to lean individuals[114], although no data on vWAT was given. Here we compared lipid turnover rates in scABD and OM fat depots across a wide range of BMIs. Associations between lipid age and anthropometry were analysed by linear regression. A positive correlation between lipid age and BMI was found in both fat depots and waist-to-hip ratio (WHR; higher waist-to-hip value indicates central body fay accumulation) also correlated negatively with lipid age, but only in the OM fat depot. Even in the significant correlations, this association were weak, indicating that there is no simple linear relationship between lipid age and total or regional body fat mass. As such, subjects were broken into categories based on BMI, body fat mass, or fat mass distribution, for subsequent analyses.

Based on BMI, subjects were broken down into 3 categories; lean, overweight/obese, morbidly obese. The mean lipid age in the scABD depot was 0.6 years higher in the overweight/obese group compared to lean individuals. No subsequent increase in lipid age was found in the excessively obese group (BMI $\geq$ 40 kg/m^2). Lipid age in the OM depot was not influenced by BMI, except in the excessively obese group, where the lipid age was increased by 0.7 years compared to the overweight/obese group. The increase in lipid age with BMI in both depots was not influenced by sex. A negative correlation between vWAT lipid age and noradrenaline-stimulated lipolysis suggested that the lipid removal rate (K_{out}) primarily reflects catecholamine-induced lipolysis in vWAT depot (as reported previously for scWAT[114]). Taken together, lipid turnover remains stable in vWAT over a broad range of BMIs and is only reduced in excessive obesity, mainly due to a decreased lipid removal rate. In scWAT, a similar manner of reduced lipid turnover was observed, however, it occurred earlier already in the overweight state.

To explore the influence of body fat distribution on lipid turnover, subjects were subdivided into tertiles based on android/gynoid fat mass or visceral/total fat mass as indications of fat distribution. Although android/gynoid and visceral/total fat mass were not correlated with scABD lipid turnover, individuals with central obesity (high android/gynoid ratio) or pronounced visceral obesity (high visceral/total fat mass ratio) had average 0.7 years younger

lipid age and 1 kg/year higher lipid storage rate in the OM fat depot compared to those with peripheral obesity or little visceral fat mass. Therefore, central or visceral obesity is linked to increased lipid turnover in vWAT. We further investigated the relationship between lipid age and body fat distribution by selecting obese individuals where detailed information on scABD and vWAT fat mass was known. The subjects were then subdivided into quartiles based on their lipid age. Body fat mass and distribution indexes were compared between the groups with the highest and lowest quartiles of mean lipid age. This analysis showed that no significant difference was found in the scABD depot, whilst a low OM lipid age associated with increased WHR and central/visceral fat accumulation.

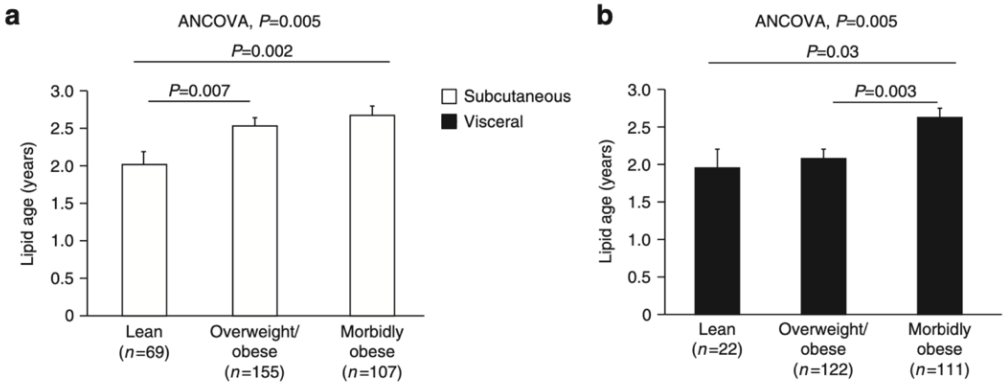

Figure 4.1. Lipid age in different BMI groups in **a**) scABD and **b**) OM fat depots.

pronounced visceral obesity (high visceral/total fat mass ratio) had averagely 0.7 years younger lipid age and 1 kg/year higher K_{in} in the OM depot compared to those with peripheral obesity or little visceral fat mass. Therefore, central or visceral obesity is linked to increased lipid turnover in vWAT. We further tested the relationship between lipid age and body fat distribution by subdividing obese individuals according to lipid age in two depots. Body fat mass and distribution indexes were compared between the groups with highest and lowest quartiles of mean lipid age. We showed that no significant difference was found in the scABD depot, while low OM lipid age was associated with increased WHR and central/visceral fat accumulation.

To directly investigate regional differences in lipid turnover between the scABD and OM fat depots, we compared lipid age in individuals where we were able to obtain adipose tissues biopsies from both depots from the same individual. Lipid age was slightly higher (approx. 0.3 years) and lipid storage rate much higher (approx. 0.7 kg/year) in the scABD compared to the OM fat tissue.

Finally, we investigated the relationship between adipocyte size and lipid age in metabolically healthy and unhealthy obese individuals. No correlation between adipocyte size and lipid age was found. As expected, unhealthy obese individuals had fewer but larger adipocytes in the

scABD depot. However, when subjects were grouped based on adipocyte size, lipid age was increased only in the small adipocytes of unhealthy compared to healthy subjects. No such difference was found in subjects with large adipocytes.

4.1.2. Discussion and future perspectives

In this study, we showed for the first time that *in vivo* lipid handling in scWAT and vWAT differs in humans. Lipid age in both the scABD and OM depots increased with body fat mass, however, with depot-specific traits (**Figure 4.1**). Results suggested that the scABD depot reaches its maximal storage and release capacity already early in the development of obesity, which may explain why ectopic lipid deposition in the liver is already observed following only a moderate body weight gain in non-obese individuals. In contrast, vWAT maintained a stable lipid turnover rate over a wide range of body fat mass, and was only reduced in excessive obesity. The reduced lipid turnover rate in both fat depots was mainly attributed to a diminished capacity of lipid removal.

Body fat distribution selectively impacts lipid turnover dynamics in the OM depot, and central/visceral obese subjects were found to have an increased OM lipid turnover rate. This is attributed to a combination of increased capacity to store (K_{in}) and remove (K_{out}) lipids. The higher vWAT lipid turnover in central/visceral obese subjects may contribute to an increase in portal FA fluxes and facilitate the adverse metabolic profile associated with high vWAT mass. Moreover, the higher average lipid age of scABD adipose tissue, compared to OM adipose tissue, is attributed to a higher lipid storage capacity, and could be an important reason why scWAT serves as the main lipid storage site in obesity.

One would expect that unhealthy obese individuals with fewer, larger scABD adipocytes would have a lower lipid turnover (and thus higher lipid age). However, this was not the case, as we observed reduced lipid turnover only in unhealthy obese individuals with relatively small adipocytes. This suggests that ectopic lipid deposition may not solely result from an insufficient increase in scWAT adipocyte size and/or number (i.e. in unhealthy obese subjects with fewer but large adipocytes) but also a diminished capacity for lipid turnover before adipocytes reach their maximum storage capacity.

Our study has some limitations. Firstly, we only investigated Caucasians and as such racial variations in regional lipid turnover cannot be ruled out, and should be explored before broad statements about depot differences in humans can be concluded. Secondly, the femoral fat depot has been shown to be protective from the metabolic complications of obesity[42, 86]. We did not sample adipose tissue from this depot as the study was designed long before this information was available. Extreme diets, such as exclusive marine diets, may lower the intake of ^{14}C incorporated into lipids. However, after the year 2000, when most of the samples were collected, the atmospheric ^{14}C levels have been low, implying the effects of exclusive marine diets would

be negligible[125]. In addition, these diets in urban Sweden (where included subjects were recruited) are rare. ^{14}C content differs between fresh and frozen food products, resulting in a "food lag". We show that mean lipid age is significantly older than blood taken from the same individuals, suggesting that estimated lipid age is not merely a result of a food lag. Of note however, these methodological issues are less relevant as this study was focused on intra-individual comparisons of scWAT and vWAT.

In summary, scWAT has a greater storage capacity than vWAT, but attains its maximal level as early as in the overweight state, which may increase the risk for lipid spill over and ectopic fat deposition in obesity. Body fat distribution only impacted on vWAT lipid turnover, where both lipid storage and removal rates were increased in central/visceral obesity. This helps explain the strong link between central body fat accumulation and metabolic complications. Finally, inappropriate scABD lipid handling in small adipocytes may be a determinant of an unhealthy obese phenotype. However, further studies combining detailed clinical phenotyping with regional lipid turnover measurements are needed to confirm the role of regional lipid turnover in metabolic disease.

4.2. Paper II

Adipose Lipid Turnover and Long-term Changes in Body Weight

4.2.1. Summary of results

Cross-sectional studies have shown that lipid removal rate decreases in obesity[114, 126]. Whether lipid turnover is constant over our life span or changes during long-term weight change is unknown. We performed a longitudinal investigation, following subjects for up to 16 years, to elucidate whether there are changes in lipid turnover during aging. In a different cohort, we followed patients for 5 years after significant weight loss by bariatric surgery.

In cohort 1, lipid age in scABD increased significantly by 0.6 years over an average timespan of 13 years, despite large inter-individual variation. Lipid age did not correlate with subject age. Furthermore, increased lipid age over time (Δ lipid age), which reflects a decreased in lipid removal rate, was not correlated with a subject's starting age. Body weight change over time and Δ lipid age were not correlated, with the Δ lipid age not differing between weight gainers (≥7% weight gain) and weight losers (≥7% weight loss). Taken together, the lipid removal rate in scWAT slows down in adults with age. This reduced lipid removal rate leads to an accumulation of body fat over time unless compensated by a decrease in lipid uptake (by proxy, food intake). Individuals with no change in lipid uptake during the examination period demonstrated approximately a 20% increase in body weight (**Figure 4.2**, where the regression line crosses zero). Other factors, such as physical activity and dietary composition, may also influence lipid accumulation in WAT[127, 128]. However, there was no correlation between lipid age and physical activity level or food composition change over time, based on participants' self-reports.

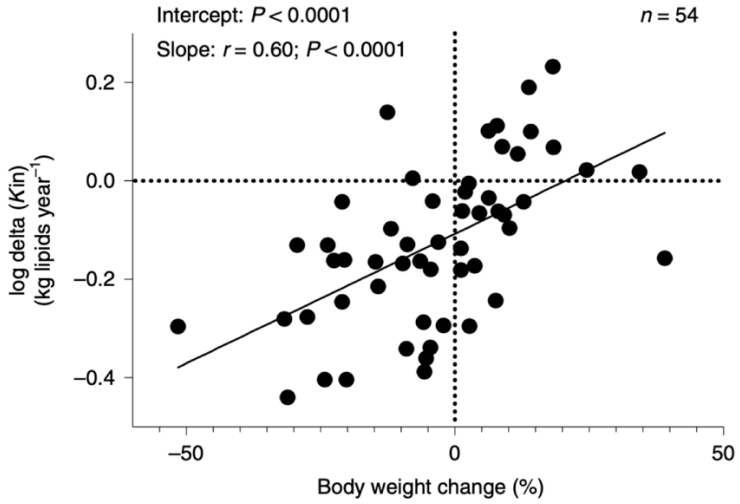

Figure 4.2. Percentage of change in body weight versus change in lipid uptake ($\log_{10}(K_{in})$) over time.

In cohort 2, we investigated severely obese individuals who underwent gastric bypass surgery. These patients demonstrated a significant and sustained decrease in BMI over 5 years after surgery. However, no change in lipid age was found, indicating that weight loss was not driven by an alteration in the lipid removal rate. There was a significant decrease in lipid uptake (K_{in}) 5 years after surgery, suggesting net lipid uptake is the main determinant driving weight loss following surgery. Notably, we showed a significant correlation between weight loss over 5 years (Δ BMI) and lipid age at year 0, with the largest Δ BMI observed in patients with the highest initial lipid age, independent from initial BMI. When patients were grouped into tertiles based on their ability to maintain weight loss 5 years post-surgery (weight rebounders, tertile 1 or weight-stable, tertiles 2 and 3), the only parameter that correlated with weight stability during 5 post-surgery years was lipid age prior to surgery (t = 0). Surprisingly, lipid age was highest at t = 0 in the weight-stable patients compared to weight rebounders. (**Figure 4.3 a and b**). Although average lipid age remained unchanged across all tertiles, if weight-stable and weight rebounding individuals were examined separately (i.e. tertile 1 versus tertiles 2 and 3), lipid age decreased in the weight-stable patients and increased in the weight rebounders, suggesting that decreased lipid age, and therefore increased lipid removal rate, following substantial weight reduction contributed to successful weight loss maintenance.

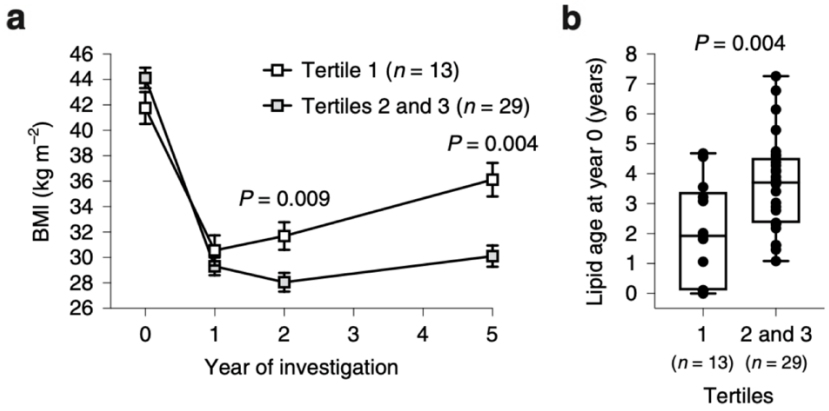

Figure 4.3. **a**) BMI at year 0, 1, 2 and 5, with patients divided into weight rebounders (tertile 1) and weight stable (tertiles 2 and 3) groups based on the change in BMI between years 2 to 5. **b**) Initial lipid age (at year 0) between weight rebounder and weight stable groups.

As age- and weight-related changes in lipid turnover may lead to compensatory ectopic lipid deposition in the liver from vWAT, we assessed fatty liver index and total vWAT mass in a second cohort. No association between lipid age change over time and fatty liver index or vWAT mass was observed. It has been shown that increased adipocyte size leads to adipose inflammation and impaired lipid storage and removal capacities[81]. In both cohorts, adipocyte size decreased while adipocyte number increased, therefore supporting the hypothesis that a hyperplastic adipose tissue is associated with better metabolic outcomes.

As energy expenditure and lipid oxidation also regulate fat mass, we compared lipid age with results from indirect calorimetry at baseline in both cohorts. Neither resting energy expenditure nor respiratory quotient correlated with lipid age, suggesting lipid removal rate is another important and independent regulator of body fat mass.

4.2.2. Discussion and future perspectives

Previous studies have shown that the major driver for obesity is calorie intake, not energy expenditure[129]. Our results add an extra dynamic to this energy balance theory.

In cohort 1, we found a reduction in lipid removal rate over time leads to weight gain unless compensated for by reducing caloric uptake. In cohort 2, we showed that lipid age prior to weight loss was higher in the weight-stable group compared to weight rebounders. This indicates that individuals with a lower lipid removal rate before weight loss may have larger window for increasing their lipid removal, compared to those with already higher removal rates before weight loss. Therefore, lipid removal rates may influence the maintenance of weight loss and therefore be an important determinant for long-term weight loss.

Although currently there is no pharmacological agent targeting lipid turnover in adipose tissue, it is possible to modify catecholamine-induced adipocyte lipolysis, which plays a central role in lipid removal rate and lipid age. For instance, endurance exercise training enhances adipocyte lipolytic activity[127, 130]. Sympathoadrenal activity decreases with age and may result in a slowed lipid turnover rate[131]. In line with this, catecholamine-induced lipolysis *in vivo* is impaired in elderly individuals and could be a potential target for obesity treatment. In summary, this study provides a new perspective on long-term weight changes in adult humans.

4.3. Paper III

The Dynamics of Fat Cell and Lipid Turnover in Omental and Subcutaneous Adipose Tissue in Adult Humans

4.3.1. Summary of results

Despite the strong link between visceral adiposity and metabolic disease, little is known about adipocyte turnover and how it associates with fat accumulation in vWAT. To investigate whether differences in adipose tissue turnover, including both adipocyte and lipid, contribute to regional differences in the distribution of body fat, we examined the age of fat cells and lipids from the scABD and OM depots in paired biopsies from the same individual.

The average age of OM adipocytes was 10.9 ± 5.4 years (mean ± s.e.m.). This was significantly older than that measured for scABD adipocytes (8.6 ± 4.4 years), which is in line with our results for a previous study[65]. There was a significant positive correlation between scABD and OM adipocyte age; such that if subjects had a high adipocyte age in the scABD depot, they were also likely to have a high adipocyte age in the OM depot. Apart from regional differences, using multivariate regression analysis we also found that a subject's age, sex and BMI significantly and independently influenced average adipocyte age and thus the adipocyte death rate. Average adipocyte age increased with subject age, indicating a decrease in adipocyte death rate with aging. Females had a lower adipocyte death rate compared to males in both the scABD and OM depots, and adipocyte death rate also decreased with increasing BMI, irrespective of depots.

We determined lipid age alongside mean adipocyte age in a cohort of 53 subjects for whom both scABD and OM fat tissue was collected. No significant difference was found between scABD and OM lipid age (3.1 ± 0.9 years vs. 3.2 ± 1.1 years), indicating no regional differences in lipid removal rate, across the BMI range studied. A sex-dependent effect on lipid age was observed, with lipid age in females significantly higher than in males. Subject age also significantly correlated with lipid age, indicating (in accordance with the findings in scABD depot in **Paper II**) that lipid turnover decreases with age. No effect of BMI on lipid age was observed. Most subjects in this study, however, were morbidly obese so potential differences between lean and obese subjects (as reported in **Paper I**) cannot be ruled out.

Clinical variables such as sex, age and BMI did not have equal impacts on the dynamics of the adipose turnover in obesity. We found that sex had the largest effect on both fat cell and lipid age.

4.3.2. Discussion and future perspectives

Adipocyte and lipid turnover are crucial contributors to the size and distribution of the fat mass[65, 114]. We demonstrate that the differences in adipose tissue turnover between scWAT and vWAT

may contribute to differences in regional fat distribution. Furthermore, we elucidated that sex-, age- and BMI-dependent differences in adipose tissue turnover contribute to fat mass regulation.

Until now, no direct information has been available concerning fat cell turnover dynamics in omental adipose tissue; one of the major visceral fat depots in humans that is strongly link to metabolic disease. We report here that mean adipocyte age is higher, and thus the death rate is lower, in the OM compared to scABD fat depot. If the fat mass is in a steady state, this would equate to a decreased adipocyte generation rate in the OM depot. We have shown previously that a low adipocyte generation rate is associated with adipose tissue hypertrophy, whereas a high generation rate is associated with adipose tissue hyperplasia[74]. Therefore, the slower turnover rate of adipocytes in the OM depot may represent a limitation in the plasticity of vWAT to accommodate an increase in energy intake. This then predisposes OM fat cells to respond by hypertrophy and increases the risk of ectopic lipid deposition once this limited storage capacity is exhausted. This hypertrophic state is known to be linked to an adverse metabolic profile. As shown in **Paper II**, OM adipose tissue maintains a constant lipid turnover rate across a wide range of body fat levels, which is only reduced in excessive obesity. scABD adipose tissue showed the same pattern, although lipid turnover was already attenuated early in the overweight state. Given the majority of subjects in the present study were morbidly obese, our finding that lipid removal rate was not significantly different between OM and scABD adipose depots may reflect a lack of difference in lipid turnover in severely obese individuals (BMI $\geq$ 40), as found in **Paper I**. Taken together, these results show that scABD adipose tissue has an increased capacity for cell turnover and, in the non-morbidly obese state, an increased capacity to store lipid compared to OM adipose tissue. Such differences may underlie the healthy expansion of scABD fat, which acts as a safe storage site for TG. Conversely, the reduced capacity of OM adipose tissue to undergo hyperplastic expansion in times of lipid excess may promote OM adipocyte hypertrophy and the associated metabolic complications.

Substantial differences at multiple levels of metabolic control have been reported between males and females, including the amount and distribution of adipose tissue[87, 88, 102, 132, 133]. We report here that both average adipocyte and lipid age are higher in females than in males. The slower death rate of fat cells and reduced lipid removal rate in females may account for the higher percentage of total body fat generally observed in females[87]. Sexual dimorphism in body fat mass and distribution found in early puberty, as well as the change of body compositions in postmenopausal women, have brought much attention to the involvements of sex hormones in adipose tissue biology[87, 88]. Sex hormones mediate their biological actions through nuclear receptors on both APs and mature adipocytes, however, many of the regulatory mechanisms remain unclear. A lower adipocyte death rate in females may be due to the protective effects of estrogen against apoptosis in both APs and mature adipocytes[90, 134], potentially contributing to a higher mean adipocyte age and higher number of fat cells in females. These characteristics may provide females (at least in the pre-menopausal state) more flexibility to accumulate

energy and better equip them for the energy demands of pregnancy and lactation. Along with reduced adipocyte death rate, lipid removal rate is also decreased in females, which may be attributed to a lower adipocyte lipolysis activity in females as reported previously[135-137]. A significant difference in lipid age between females and males was not reported in **Paper I**, which may be due to a poorer representation of males in the previous study (15% males) compared to this study (36% males).

Body fat mass increases throughout life, with the increase distributed preferentially to the abdominal region compared to lower body fat regions[138, 139]. This change is associated with an increased risk of metabolic disease in the elderly. In line with this, we found that the death rate of adipocytes was reduced with aging in the both scABD and OM depots, which in turn may promote fat cell accumulation in the abdominal region. Aging had been found to decrease AP proliferation and differentiation rates in elderly individuals and increase AP senescence, further reducing the proliferation capacity of AP and hence promoting hypertrophic adipose tissue expansion[139-141]. Therefore, a decrease in the death rate of mature adipocytes, along with an impaired production rate of new adipocytes, may contribute to a slower adipocyte turnover and limited plasticity of adipose tissue for healthy expansion with aging. Changes in lipid turnover with age were also reported in **Paper II**, where the scABD lipid removal rate decreases during aging. We confirm and extend these results in the current study to OM adipose tissue as well. Taken together, we demonstrated that a reduced adipocyte death rate and lipid removal rate, together promote an increase in abdominal fat mass with age.

When adjusted for depot, sex and age, we found a high BMI was associated with a high adipocyte age and low adipocyte death rate. This would, over time, result in an increase in fat cell number in obese compared to lean individuals. In a cross-sectional study, where total fat cell number in lean and obsess adults was calculated, obese individuals were shown to have a two to three-fold increase in number, compared to lean individuals[65]. In **Paper I**, differences in the lipid removal rate between the scABD and OM fat depots was lost in morbid obesity (**Figure 4.1**), which may explain why in this study we did not see an effect of BMI on lipid removal rate (56 out of 66 subjects were obese in this study). Unfortunately, there are clear challenges in obtaining sufficient amount of fat tissue required for ^{14}C measurement from lean individuals, let alone from both the scABD and OM fat depots within the same individual. Therefore, it is a limitation of this study that the examined population is skewed towards the morbid obesity.

In summary, we provide the first comprehensive data about the age of omental fat cells in humans. Furthermore, we show that alterations in the adipocyte death rate and lipid removal of OM and scABD fat depots contribute to differences in regional adiposity. We also demionstrate that sex and age are important determinants of adipose tissue turnover dynamics. A better understanding of the factors governing body fat accumulation and distribution may lead to the

development of more targeted treatment strategies for individuals with, or at high risk of, metabolic disease.

5. CONCLUDING REMARKS

The synthesis and breakdown of adipocytes and lipids control the number and size of fat cells, respectively, and thus play a central role in obesity development. In this thesis, I present how adipocyte and lipid turnovers differ in scWAT and vWAT, in obese and lean individuals, and how this contributes to total fat mass accumulation and distribution. Moreover, how aging and sex affect adipose tissue turnover is discussed.

The reduced adipocyte turnover rate in OM adipose tissue demonstrates the limited capacity of vWAT to expand by means of hyperplasia, predisposing OM fat cells to respond to excess energy by hypertrophic expansion, and thereby promoting ectopic lipid deposition. Lipid removal rate decreased both in scWAT and vWAT as BMI increased, however, the removal rate in vWAT remained relatively stable over a broad range of BMIs and was only reduced in excessive obesity. On the other hand, a reduced lipid removal rate in scWAT occurred already earlier in the overweight state. These results suggest that scWAT is the "frontline" storage space at obesity onset, and once it reaches the limits of expandability, ectopic lipid deposits into the vWAT fat. The limited expandability of vWAT may promote the hypertrophic expansion of visceral adipocytes and contribute to ectopic lipid deposition into secondary storage sites, such as the liver and muscles.

We showed that the removal rate of adipocytes decreases with aging in both scABD and OM fat depots. This contributes to an increase in the fat mass in the abdominal region and subsequently central obesity. In line with this, the scABD lipid removal rate also decreases with aging. We determined that a failure to reduce lipid intake (which serve as a proxy for food intake) over a 13year period results in approximately a 20 percent increase in body weight. Interestingly, central obese individuals showed a reduced lipid removal rate, indicating an interaction between adipocyte and lipid turnover. We also uncovered fundamental differences between females and males in the ways that they turnover their lipids and fat cells, which may contribute to the higher body fat percentage generally observed in females.

Based on our results, differences in adipocyte and lipid turnover present a potential treatment target for mitigating the metabolic complications associated with obesity and fat mass distribution. Understanding the turnover dynamics of adipose tissues in humans gives clearer insights into the factors regulating the fat mass in humans. This thesis identifies the factors affecting adipose tissue turnover and provide new clinical perspectives on obesity treatment.

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